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Rezk PE, Zdenka P, Sabnekar P, Kajih T, Mata DG, Wrobel C, Cerasoli DM, Chilukuri N.

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US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDR-I
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

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RESEARCH ARTICLE

An *in vitro* and *in vivo* evaluation of the efficacy of recombinant human liver prolidase as a catalytic bioscavenger of chemical warfare nerve agents

Peter E. Rezk, Pierre Zdenka, Praveena Sabnekar, Takwen Kajih, David G. Mata, Chester Wrobel, Douglas M. Cerasoli, and Nageswararao Chilukuri

Physiology & Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA

Abstract

In this study, we determined the ability of recombinant human liver prolidase to hydrolyze nerve agents *in vitro* and its ability to afford protection *in vivo* in mice. Using adenovirus containing the human liver prolidase gene, the enzyme was over expressed by 200- to 300-fold in mouse liver and purified to homogeneity by affinity and gel filtration chromatography. The purified enzyme hydrolyzed sarin, cyclosarin and soman with varying rates of hydrolysis. The most efficient hydrolysis was with sarin, followed by soman and by cyclosarin {apparent k_{cat}/K_m [(1.9 ± 0.3), (1.7 ± 0.2), and (0.45 ± 0.04)] $\times 10^5\,M^{-1}\,min^{-1}$, respectively}; VX and tabun were not hydrolyzed by the recombinant enzyme. The enzyme hydrolyzed P (+) isomers faster than the P (−) isomers. The ability of recombinant human liver prolidase to afford 24 hour survival against a cumulative dose of $2 \times LD_{50}$ of each nerve agent was investigated in mice. Compared to mice injected with a control virus, mice injected with the prolidase expressing virus contained (29 ± 7)-fold higher levels of the enzyme in their blood on day 5. Challenging these mice with two consecutive $1 \times LD_{50}$ doses of sarin, cyclosarin, and soman resulted in the death of all animals within 5 to 8 min from nerve agent toxicity. In contrast, mice injected with the adenovirus expressing mouse butyrylcholinesterase, an enzyme which is known to afford protection *in vivo*, survived multiple $1 \times LD_{50}$ challenges of these nerve agents and displayed no signs of toxicity. These results suggest that, while prolidase can hydrolyze certain G-type nerve agents *in vitro,* the enzyme does not offer 24 hour protection against a cumulative dose of $2 \times LD_{50}$ of G-agents in mice *in vivo*.

Keywords

Adenovirus, butyrylcholinesterase, catalytic bioscavenger, chemical warfare nerve agents, human liver prolidase, *in vivo* delivery

History

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Introduction

Chemical warfare nerve agents (NAs) are among the deadliest compounds known to man, and they pose a great threat to military personnel and civilians alike. Their toxicity stems from their ability to inhibit acetylcholinesterase (AChE), which is responsible for the breakdown of the neurotransmitter acetylcholine (ACh) (De Candole et al., 1953; Yokoyama et al., 1998). The resultant accumulation of ACh at cholinergic synapses produces an acute cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculations, behavioral incapacitation, muscular weakness, and convulsions culminating in death by respiratory failure (Brimblecombe, 1977). Developing a viable treatment for these toxicities has been a scientific challenge, and different therapeutic approaches are being investigated. Currently, treatment of NA intoxication involves the use of a combination of pharmacological therapies to counteract the effects of NA-induced inhibition of AChE. This treatment includes cholinolytic drugs such as atropine sulfate, a competitive antagonist of the acetylcholine receptors (Shih & McDonough, 1999; Shih et al., 2007), oximes such as pyridinium-2-aldoxime (2-PAM) to restore the activity of NA-inhibited AChE, (Gray, 1984; Haigh et al., 2005; Koplovitz & Stewart, 1994) and anticonvulsant drugs such as diazepam to control NA-induced tremors and seizures (Dickson et al., 2003; Marti et al., 1985; Shih et al., 2007). Although these treatments effectively prevent NA lethality, they do not prevent performance deficits, loss of consciousness, or permanent brain damage (Dirnhuber et al., 1979).

An alternate approach is the use of bioscavenger proteins, or molecules that circulate in the blood stream and detoxify NA, before they gain access to AChE. There are two types of bioscavengers: stoichiometric and catalytic. With the stoichiometric type, one molecule of the

Address for correspondence: Nageswararao Chilukuri, Physiology & Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010, USA. Tel: 410-436-5106. Fax: 41-436-8377. E-mail: Nageswararao.chilukuri.civ@mail.mil

bioscavenger neutralizes one molecule of the NA. Plasma-derived human butyrylcholinesterase (HuBChE) is the leading candidate of this type (Broomfield et al., 1991; Lenz et al., 2005). In multiple animal models, native or recombinant human BChE alone was shown to provide protection against all types of NA (Huang et al., 2007; Lenz et al., 2007). However, a large dose of the enzyme is needed to afford protection. For example, it is estimated that a dose of 200 mg of the pure enzyme is necessary to protect a 70 kg individual against $2 \times LD_{50}$ doses of soman (Wolfe et al., 1992).

With catalytic bioscavengers, one molecule of bioscavenger can hydrolyze hundreds or thousands of molecules of NA. Therefore, catalytic bioscavengers have inherent advantages over stoichiometric bioscavengers: (1) they are not consumed in the process of detoxifying NA, and (2) they work at low concentrations, and therefore significantly less protein is required for protection against multiple LD_{50}'s of NA (Masson, 2011). Human prolidase is one of the candidate catalytic bioscavengers under evaluation for possible development as a prophylactic against NA (diTargiani et al., 2010; Wang et al., 2006).

Prolidases are ubiquitous in nature and have been isolated from a variety of mammalian tissues (Sjostrom et al., 1973). Different isoforms of prolidase exist and have been isolated and characterized from human liver, kidney, and skin (Myara et al., 1994; Wang et al., 2005). Human prolidase is composed of two identical subunits of MW 54 kDa with an optimum activity at pH 7.8 and 37–50 °C, requiring Mn^{2+} ions for full activity (Endo et al., 1982; Kokturk et al., 2002). The primary function of prolidase is to hydrolyze various dipeptides containing proline or hydroxyproline at the C-terminus, with the preferred substrate for prolidase being the Gly-Pro dipeptide (Endo et al., 1982; Myara et al., 1994). Other dipeptides hydrolyzed by prolidase include Ala-Pro, Met-Pro, Phe-Pro, Leu-Pro and Val-Pro. Thus, prolidase plays a crucial role in the complete degradation of proline-rich proteins such as collagen of the skin (Palka, 1996; Palka et al., 1996).

Human prolidase exhibits sequence homology with bacterial organophosphorous acid anhydrolases (OPAA), a group of enzymes that hydrolyze many organophosphorous compounds (OP) including NA (Hill et al., 2001; Letant et al., 2005). Because of this similarity, native and recombinant human liver, kidney and skin prolidases were investigated for their NA hydrolyzing activity *in vitro* by the use of an AChE back titration assay, which is an indirect method of measuring NA hydrolysis. It was found that native and recombinant human skin, kidney, and liver prolidases protected AChE from inhibition by NA. While the liver enzyme hydrolyzed DFP and all G-agents, kidney and skin prolidases hydrolyzed DFP and sarin only (Costante et al., 2012). Studies on the ability of human skin, liver, or kidney prolidase to offer protection against G-agents *in vivo* in animals have been lacking.

In this study, we used recombinant adenovirus containing the gene for human liver prolidase (Ad-prolidase) to produce a large amount of recombinant human liver prolidase (rhProlidase) in mouse liver. rhProlidase was purified to homogeneity from the liver and characterized for its ability to hydrolyze NA by a direct NA hydrolysis assay. The fastest rate of hydrolysis was observed with sarin (GB), followed by cyclosarin (GF), and soman (GD); VX and tabun (GA) were not hydrolyzed by the recombinant enzyme. Ad-prolidase injected mice also contained 30-fold higher levels of rhProlidase in their blood compared to mice injected with a control virus. The mice with such elevated blood levels of rhProlidase failed to gain 24 hour protection against a $2 \times LD_{50}$ cumulative dose of cyclosarin, sarin, or soman.

Materials and methods

Materials

All chromatography columns were purchased from GE Healthcare (Piscataway, NJ). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce ThermoScientific (Rockford, IL). Gly-Pro, butyrylthiocholine (BTC), 5-5′-dithiobis (2-nitrobenzoic acid) (DTNB), diisopropylfluorophosphate (DFP) and all other analytical chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Precast SDS-PAGE gels were purchased from BioRad Inc. (Hercules, CA). Nerve agents *O*-ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun, GA), isopropoxymethylphosphoryl fluoride (sarin, GB), cyclohexyl methylphosphofluoridate (cyclosarin, GF), *O*-pinacolyl methyphosphonofluoridate (soman, GD), and *O*-ethyl S-2-*N*,*N*-diisopropylaminoethyl methylphosphonothiolate (VX) were obtained from the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. The purity of NA was >98.5% as determined by ^{31}PNMR.

Recombinant adenoviruses

Adenoviruses expressing rhProlidase (Ad-prolidase), and mouse butyrylcholinesterase (Ad-BChE), as fusion proteins with a 6 × histidine tag at their C-terminus, were produced as described before (Aleti et al., 2013). A null adenovirus lacking the human liver prolidase gene but otherwise identical Ad-prolidase or Ad-BChE was purchased from Welgen Inc. (Worcester, MA).

Expression of rhProlidase in mouse liver

The experimental protocols were approved by the Institutional Animal Care and Use Committee (U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD), and research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. All work adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 Edition. For these studies Swiss-Webster mice (male, 20–25 g body weight) were housed at 20 °C and were provided food and water *ad libitum*. Ad-prolidase (2×10^{11} viral particles/per mouse) was injected via the tail vein, and 4 to 5 days later animals were euthanized and livers were collected. Tissue was homogenized in ice cold buffer containing 50 mM potassium phosphate, pH 7.4, and 0.5% Tween 20, and centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was removed, assayed for prolidase activity and protein concentration and stored frozen at −80 °C until

use. Livers were collected from 5 animals for purification of the recombinant enzyme.

Purification of rhProlidase

The purification of rhProlidase from livers of mice injected with Ad-prolidase was accomplished using two–step chromatography involving affinity and gel filtration and was performed using Fast Protein Liquid Chromatography (FPLC, Akta Explorer, GE Healthcare). The supernatant from liver homogenate was loaded on a nickel-sepharose high performance beads packed column (5 mL) that had been equilibrated with 15 column volumes (CV) of buffer A (20 mM Tris-HCl, pH 7.0, containing 15 mM imidazole, 500 mM NaCl). The column was washed with 10 CV of buffer A until the absorbance (OD280) was dropped below 0.05 and was eluted with 20 CV linear ascending gradient of 400 mM imidazole in buffer A at a flow rate of 1 mL per min. The fractions were subjected to SDS-PAGE and DFP hydrolysis assay for pooling and subsequent gel filtration chromatography. The fractions containing DFP hydrolysis activity were concentrated using an Amicon Centrifugal filter with a 10-kDa cut off (Millipore Corp., Billerica, MA) and applied to Superdex HiLoad 16/60 column equilibrated with 20 mM Tris-HCl buffer, pH 7.4, with 500 mM NaCl. The enzyme was eluted at a flow rate of 0.5 ml/min. Fractions containing DFP-hydrolyzing activity were pooled and subjected to SDS-PAGE to check the purity of the recombinant enzyme. Two batches of the enzyme were made and pooled together for use in nerve agent hydrolysis assays.

Prolidase activity assay

Mouse plasma or tissue samples were tested for dipeptidase activity by the method of Myara et al., (Myara et al., 1982). Samples were diluted to 100 µL of 50 mM Tris/HCl, pH 7.4, containing 1 mM $MnCl_2$ and were preincubated for 24 h at 37 °C. Samples were then mixed with 100 µL of 94 mM glycyl-proline in 50 mM Tris/HCl, pH 7.4, containing 2 mM $MnCl_2$. After 30 min of incubation, the enzyme reaction was stopped with the addition of 1 mL of 0.45 M trichloroacetic acid, and the supernatant was used for proline determination as described previously (Myara et al., 1982). One unit of prolidase activity represents the amount of enzyme that catalyzes the hydrolysis of one µmole of substrate, per minute at 37 °C.

DFP hydrolysis assay

Prolidase activity was also determined by measuring the hydrolysis rate of DFP at 25 °C. The hydrolysis of DFP releases H^+ ions that are monitored by the increase in absorbance at 422 nm, as described (Billecke et al., 1999; diTargiani et al., 2010).

Nerve agent hydrolysis

The hydrolysis assay for different G-type nerve agents was performed as described (Otto et al., 2010). The purified recombinant enzyme was incubated for 1 h at 37 °C with 100 mM MOPS, pH 8.0, and 2 mM $MnCl_2$. Then a racemic sample of each G-type nerve agents was added and incubated at room temperature. The initial concentrations of G-type nerve agent were 4.2 mM, 4.5 mM, 4 mM and 6 mM for GB, GF, GD and GA respectively. The concentration of prolidase was 178.6 nM. At different time intervals, 50 µL aliquots were removed and inactivated through extraction with an equal volume of ethyl acetate containing 50 µM DFP (internal standard). The organic layer (containing non-hydrolyzed nerve agent) was then removed and analyzed by gas chromatography/mass spectrometry (GC/MS) using an Agilent 6890 GC equipped with a 7890 MS Detector (Otto et al., 2009, 2010). Separation of the nerve agent enantiomers was achieved using a Chiral Dex$^{(R)}$ (TA) column (20 M × 250 µm × 0.10 µm) (Advanced Separations Technologies Inc., Whippany, NJ). The stationary phase was 2, 6-di-*O*-pentyl-3-*O*-trifluroacetyl g-cyclodextrin. Initially, a universal method was created for all OPs analyzed. Instrumental parameters included a helium flow rate of 1.4 mL/min, and injection port and transfer lines of 210 °C and 220 °C, respectively. The oven temperature program was 90 °C (7 min), 10°/min to 80° (5 min) and 20°/min to 1700 (2.5 min). A 1 µL injection was used with a 50:1 split ratio. A stock solution was created containing each agent and was diluted in ethyl acetate with DFP as the internal standard. Selected monitoring mode (SIM) was used for detection. Ions used were 99, 81 and 125, for GB; 99, 82 and 126, for GD; 67, 99, 137, and 67 for GF; and 70, 133, and 162 for GA. Ions for the internal standard DFP were 101 and 127. All agents and their isomers eluted in less than 20 min with complete resolution between individual stereoisomers. The non-hydrolyzed quantity of nerve agent in each sample was determined by comparison to both the DFP internal standard and the specific nerve agent standard calibration curve. Kinetic parameters (k_{cat}/K_m) were determined by fitting the data to a one-phase exponential decay defined by the equation below (Otto et al., 2010):

$$y = y_0 e^{-Kx}$$

A modified Ellman colorimetric assay was used to measure hydrolysis of VX as described elsewhere (Otto et al., 2009).

Nerve agent challenge

Challenge experiments with nerve agents GD, GB, and GF were performed at the USAMRICD. Ad-prolidase, Ad-control, and Ad-BChE viruses were injected into the tail vein of mice as described (Parikh et al., 2011). Four to nine mice were included in each group. Ad-BChE was used as a positive control since it was expected to provide protection from LD_{50} doses of all nerve agents, the protection offered based upon the activity levels of the enzyme (Parikh et al., 2011). Plasma was collected from all the mice before the virus injection and on day 5 prior to nerve agent challenge. On day 5, mice were challenged subcutaneously with the following nerve agents: 1 × LD_{50} of GD (110 µg/kg), 1 × LD_{50} of GF (110 µg/kg), and 1 × LD_{50} of GB (220 µg/kg). Mice were observed for signs of cholinergic symptoms through 1 h post-challenge. Animals that survived the first 1 × LD_{50} challenge were challenged again with another 1 × LD_{50} of the same agent at 1 h after the first challenge. Animals were observed

for 1 h for cholinergic symptoms and 24 h later euthanized as mandated by the protocol.

Other methods

The protein contents of various samples were measured using the standard BCA assay (Pierce Co, Rockford, IL). The purity of rhProlidase was analyzed by SDS-PAGE according to Laemmli (Laemmli, 1970). Protein bands were visualized by staining the gels using Bio-Rad Bio-safe.

Results and discussion

Expression and purification of rhProlidase

We used an adenovirus mammalian expression system to express and purify ~500 μg of a homogenous preparation of rhProlidase. In our previous investigation testing the capacity of Ad-prolidase to induce the expression of rhProlidase *in vivo* in mice, we found that 95–97% of the enzyme expressed by the Ad-prolidase is stored in the mouse liver five days after virus injection (~2.7 mg of the enzyme per gram tissue), (Aleti et al., 2013). Since, previous reports investigating the *in vitro* hydrolyzing activity of recombinant human prolidase employed *E. coli*, yeast, and insect cell expression systems; we undertook the present investigation of characterizing the *in vitro* hydrolyzing activity of various nerve agents using purified rhProlidase from a mammalian expression system. The purification scheme employing affinity (Figure 1A and B) and gel filtration (Figure 2A and B) chromatography produced a pure enzyme with no other bands visible on a protein gel.

The majority of the rhProlidase was partially purified by affinity chromatography using high-performance nickel affinity resin. As shown in Figure 1(A), rhProlidase bound to nickel affinity resin and eluted with an increasing concentration of imidazole at 0.2–0.25 M in five 1 mL fractions. As evidenced by SDS-PAGE (Figure 1B), the middle three peak fractions displaying DFP hydrolysis activity contained the 55 kDa rhProlidase and many other proteins, suggesting that the preparation was impure.

Further purification of rhProlidase was accomplished using a Superdex HiLoad gel filtration column. Affinity-column-purified rhProlidase was concentrated using an Amicon ultracentrifugal filter with a 10-kDa cut off membrane and the concentrate was dialyzed against 20 mM Tris-HCl buffer, pH 7.4, with 500 mM NaCl. The concentrate was then loaded on to a gel filtration column and eluted with 20 mM Tris-HCl, buffer pH 7.4, containing 500 mM NaCl. The elution profile showed three peaks; one major and two minor (Figure 2A). Analyzing these various fractions for DFP hydrolysis activity showed that the fractions at the tail end of the first peak and the beginning of the second peak contained all the enzyme activity. These fractions were pooled and visualized by SDS-PAGE to assess for the purity of the enzyme. As shown in Figure 2(B), a single protein band with a molecular weight of 55 kDa was present, suggesting that the preparation was pure.

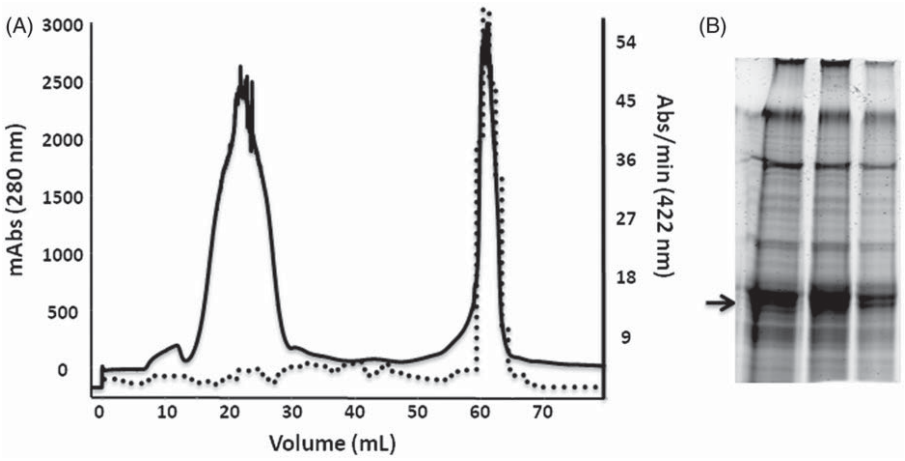

Figure 1. (A) Affinity chromatography. To purify rhProlidase, the supernatant from liver homogenate from 5 mice was run on a nickel Sepharose high performance beads packed column. Bound rhProlidase was eluted with a linear gradient 15–400 mM imidazole at a flow rate of 0.5 mL per min. The solid line is the OD at 280 nm corresponding to the protein levels in each fraction, while the dotted line represents prolidase activity determined by measuring the hydrolysis rate of DFP. (B) Fractions containing prolidase activity were analyzed by SDS-PAGE and protein bands were identified by Coomassie staining. Arrow indicates the location of 54 kDa rhProlidase.

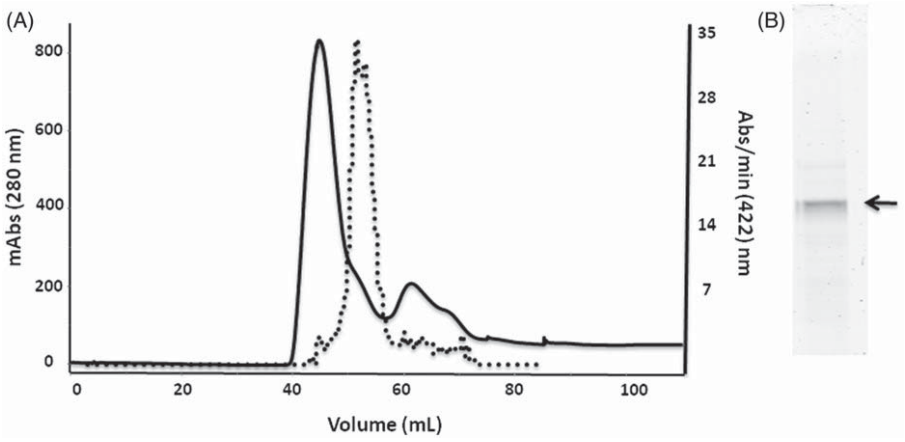

Figure 2. (A) Gel filtration chromatography: Partially purified enzyme fraction from the affinity chromatography was loaded on a Superdex HiLoad 16/60 column in a buffer containing 20 mM Tris-HCl buffer, and 500 mM NaCl and fractions of 0.5 mL were collected at a flow rate of 0.5 mL per min. The solid line represents the OD at 280 nm corresponding to the protein levels in each fraction, while the dotted line represents DFP hydrolyzing activity of the enzyme activity in the fractions. (B) Fractions containing prolidase activity were pooled and concentrated and purity of the enzyme was analyzed by SDS-PAGE. Arrow points to the enzyme purified by gel filtration chromatography.

In vitro hydrolysis of nerve agents by rhProlidase

Previous studies determining the ability of rhProlidase to hydrolyze NA utilized an indirect assay in which the residual unhydrolyzed NA in the reaction mixture was titrated with AChE, and the uninhibited AChE was measured by residual agent Ellman assay (Costante et al., 2012; diTargiani et al., 2010). In addition, these studies used rhProlidase from *E. coli*, insect cell, or yeast mammalian systems. We used the adenovirus mammalian expression system, which is known to produce recombinant human proteins that are similar to native proteins in terms of post-translational modifications. We also used a GC/MS assay that is a direct measure of the nerve agent hydrolysis. Finally, we used a chiral DEX® (TA) column to separate the P (+) and P (−) isomers of each NA to measure their respective hydrolysis rates. As shown in Figure 3 and Table 1, the ratio of P (−)/P (+) is less than 1 (GD, 0.7; GF, 0.8; and GD 0.5) suggesting that rhProlidase exhibits greater activity against the P (+) isomer compared to the P (−) isomer (Benschop et al., 1984). Overall, rhProlidase hydrolyzed NA with varying rates; the most efficient hydrolysis was observed for GB followed by GD and GF {apparent k_{cat}/K_m $[(1.9 \pm 0.3)$, (1.7 ± 0.2), and $(0.45 \pm 0.04)] \times 10^5\,M^{-1}\,min^{-1}$, respectively}. Somewhat of a different profile was reported for rhProlidase expressed and purified using an *E. coli* expression system (diTargiani et al., 2010). Whereas the enzyme expressed and purified from mouse liver failed to hydrolyze GA (Figure 3 and Table 1) and VX (data not shown), the enzyme expressed and purified from *E. coli* hydrolyzed GA to a certain extent. These differences could be attributed to the fact that different expression systems and different assay systems were employed in these studies. We used a mammalian expression system and a direct GC/MS assay whereas diTargiani et al. used bacterial expression system and an indirect interception assay/AChE back titration assay. Somewhat of a different profile was also observed for a partially purified human liver prolidase (Costante et al., 2012). This enzyme preparation was found to hydrolyze GA, GB, GD, and VX. VX hydrolysis was not observed for the enzymes purified from the mammalian and bacterial expression systems. The discrepancy between our results and those in the study above may be attributed to the level of purity of the enzyme. By SDS-PAGE we found that the rhProlidase used in this study was seen as a single band suggesting that it was pure whereas the enzyme preparation used in the previous study (Costante et al., 2012) was 70 to 80% pure and its gel image was not shown.

In vivo protection offered by rhProlidase

Human BChE, an enzyme which offers stoichiometric protection against all types of NA, is mostly exported into

Table 1. Catalytic parameters (apparent k_{cat}/K_m) of rhProlidase against GB, GD, and GF. Prolidase concentration in these experiments was 1.7 μM while the initial concentrations of G-type nerve agent were 4.2 mM, 4.5 mM, 4 mM and 6 mM for GB, GF, GD and GA respectively Reactions were carried out at a concentration of prolidase appropriate for the dynamic range of the GC/MS. Catalytic efficiency against GA and VX could not be determined because rhProlidase was not able to hydrolyze them. Apparent k_{cat}/K_m for the P- isomer was divided by the k_{cat}/K_m for the P (+) isomer to derive the ratio of P (−)/P (+). A less than 1 ratio suggests that the enzyme preferentially hydrolyzes the non toxic P (+) isomer.

Agent	Apparent k_{cat}/K_m ($\times 10^4\,M^{-1}\,min^{-1}$)			
	P (−) Isomer	P (+) Isomer	Racemic	Ratio of P (−)/P (+)
GB	7.8 ± 0.8	11 ± 2	19 ± 3	0.7
GF	2.0 ± 0.3	2.5 ± 0.2	4.5 ± 0.4	0.8
GD [C(−)]	2.8 ± 0.6	6.4 ± 0.6	17 ± 2	0.5
GD [C(+)]	2.6 ± 0.6	4.7 ± 0.4		
GA	Not detected			
VX	Not detected			

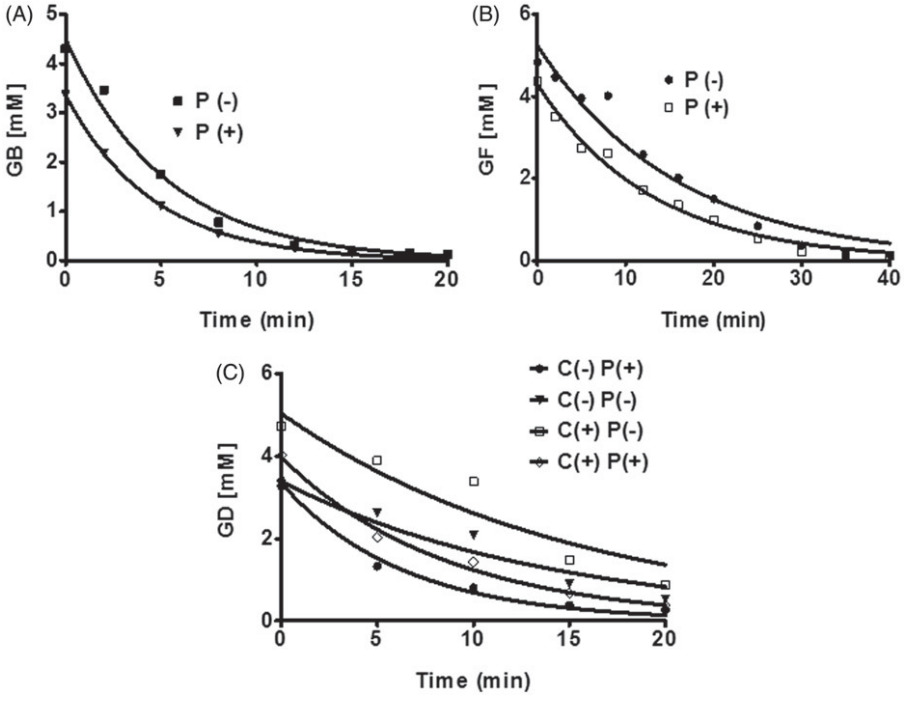

Figure 3. GB (A), GF (B), and GD (C), hydrolysis by rhProlidase. Panels show single phase exponential decay curves of the hydrolysis of different nerve agents and their respective stereoisomers. The non linear regression single phase decay curve was calculated using GraphPad Prism 4 with Plateau constraint is equal to 0. Prolidase concentration in these experiments was 1.7 μM while the initial concentrations of G-type nerve agent were 4.2 mM, 4.5 mM, and 4 mM for GB, GF, and GD respectively.

Table 2. Ability of prolidase to protect mice against nerve agents: Mice pretreated with Ad-prolidase failed to offer 24 hour protection from exposure to 47.14 nmol of GB, 18.33 nmol of GD, and 18.13 nmol of GF. In contrast, mice pretreated with Ad-BChE virus were protected from GD, GB, and GF challenges. Prolidase levels in mice treated with Ad-prolidase were 240 ± 50 units in mouse or 10.02 nmol in mouse, 30-folds higher than the prolidase levels in control virus-injected mice.

Agent	Virus injected	# of animals	Percent survival (1st LD_{50} challenge)	Percent survival (2nd LD_{50} challenge)
GB	Ad-null	9	0%	NA
	Ad-BChE	6	100%	66%
	Ad-Prolidase	8	50%	0%
GD	Ad-null	6	0%	NA
	Ad-BChE	6	100%	100%
	Ad-Prolidase	6	0%	NA
GF	Ad-null	6	16%	0%
	Ad-BChE	5	100%	100%
	Ad-Prolidase	6	33%	0

circulation. In contrast, most (95 to 97%) of the virally expressed rhProlidase was found intracellularly in the liver with the rest in circulation (Aleti et al., 2013). Nonetheless, rhProlidase levels in the circulation of mice were (29 ± 7)-fold higher than the baseline level, suggesting that this amount of prolidase might be sufficient to offer *in vivo* protection, provided the turnover rate and the binding affinity of the enzyme for NA are high. Mice were challenged with GD, GB, or GF on the fifth day after Ad-prolidase injection at which time rhProlidase levels were at peak (240 ± 50 units or ~10 nmol in mice). Mice injected with virus expressing mouse BChE were included as positive controls. On day 5, BChE expression levels were 500 to 1500-fold higher than found in the control virus-injected animals. Consecutive $1 \times LD_{50}$ doses of nerve agents were administered 1 hour apart to groups of 6 to 8 mice (Table 2). All animals in the control group died following the first $1 \times LD_{50}$ dose of GD, GB, and GF indicating that the dose of nerve agent injected may have been more than a $1 \times LD_{50}$. Time to death following one or two $1 \times LD_{50}$ doses of nerve agent was between 5 to 15 min for control virus and Ad-prolidase virus injected animals. In the case of GB, four out of eight animals survived $1 \times LD_{50}$ of the nerve agent relative to none in the control group (Table 2). In the case of mice injected with GF, two out of six animals survived a $1 \times LD_{50}$ dose of the nerve agent relative to only one animal in the control group. The surviving animals in both of these groups died following the second $1 \times LD_{50}$ of the nerve agent. These results, at best, suggest that human liver prolidase may offer partial protection against sub-lethal doses of GD and GF (Table 2). Additional experiments are necessary to confirm if prolidase indeed offers protection against sub-lethal doses of GB and GF. For this study, the measure of protection chosen was 24 hour survival against a single or cumulative $2 \times LD_{50}$ or higher dose of GD, GF, or GB. In contrast to the prolidase group, mice which received the virus responsible for BChE expression survived two sequential $1 \times LD_{50}$ doses of GD, GB, and GF and showed no signs of toxicity for the 24 hours observation period after the challenge. Collectively, these results suggest that human liver prolidase fails to offer 24 hour survival protection against $2 \times LD_{50}$ cumulative dose of G-type nerve agents.

It is unlikely that the failure of prolidase to provide 24 hour survival against a cumulative dose of $2 \times LD_{50}$ of GD, GF, and GB is due to failure of the adenovirus expression system to produce sufficient levels of enzyme in the blood. On day 5, when the mice were challenged with NA, the ratio of blood enzyme to NA concentration was ~1:2 (10.02 nmol of enzyme vs 18 nmole of GD and GF). Additionally, we have successfully used the adenovirus system previously to induce overexpression of human and mouse BChE and human paraoxonase-1 (PON1) in mice, and have achieved protection against several LD_{50} doses of nerve agents and OP compounds (Duysen et al., 2011). In the present case, it is likely that the rhProlidase scaffold is unable to afford protection due to either: (1) the enzyme has a higher affinity for the less toxic P (+) isomer, which hinders the ability of the enzyme to sequester away the more toxic P (−) isomer before onset of toxicity, or (2) the catalytic efficiencies of rhProlidase against the P (−) isomers of GD, GB, and GF are not sufficient enough to lower their blood concentration to non-lethal levels. For wild-type human prolidase to become more efficient *in vivo*, the turnover rate and binding affinity of the enzyme for nerve agents, specifically for the P (−) isomers, may have to be substantially improved through site-directed mutagenesis and/or random and rational directed evolution. Such studies have proven to be valuable in making variants of PON1 that have improved catalytic efficiencies against G-type nerve agents as well as stereochemical preference for the more toxic P (−) isomers *in vitro* and *in vivo* (Gupta et al., 2011). Recently, we have investigated the ability of these variants to provide protection against G-type nerve agents *in vivo* in the mouse model using our adenovirus expression system. The most successful PON1 variant has enhanced catalytic efficiencies against each G agents, favored the hydrolysis of P (−) isomer over the P (+) isomer, and provided symptom free 24 hour protection against super-stoichiometric doses of GA, GB, GD, and GF constituting multiple lethal doses of each agent (Mata et al., Manuscript in preparation). Taken together, it appears that a combination of catalytic efficiency and stereochemical preference towards the toxic P (−) isomer governs the ability of an enzyme to protect against nerve agent exposure.

Conclusions

Recombinant human liver prolidase was expressed in mouse liver and purified to homogeneity by affinity and gel filtration chromatography. The recombinant enzyme hydrolyzed certain G-type nerve agents *in vitro* but mice containing elevated blood levels of this enzyme did not receive 24 hour protection from a cumulative dose of $2 \times LD_{50}$ of GD, GF, and GB.

Declaration of interest

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Institute of Chemical Defense, Aberdeen Proving Ground, MD and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The authors are grateful to Dr. John McDonough, USAMRICD, for help with the animal experiments and suggestions with the manuscript.

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